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In Anticipation of a Noninvasive Immunodiagnostic Strategy for Confirmation of Sulfur Mustard Skin Exposure: The Technique for Skin Tape-Stripping

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14. ABSTRACT
Continuing efforts to develop a fieldable noninvasive immunodiagnostic strategy confirmatory for sulfur mustard (HD) skin exposure have led to exploring the potential of stratum corneum tape-stripping to eventually visualize HD-induced keratin adducts on the tape before the onset of the vesication phase of HD pathology. In preliminary experiments summarized here, selected tapes with adhered stripped cells were tested in laboratory-based study for nonspecific staining fidelity, cellular adhesion, microscopic clarity and as proof of concept for eventual in-the-field diagnostic applications. Three tapes were tested in these experiments: two double-sided optically clear nonallergenic industrial tapes identified as Y and Z and medical adhesive grade double-sided clear tape identified as A. Tapes with adherent cells were subjected to three nonspecific staining procedures derived from epoxy-embedded electron microscopy practices (methylene blue, basic fuchsin and azure II) and three established cellular permeabilization pretreatments (100% acetone, 100% methanol, Triton X-100). Staining, handling and storage trials resulted in the selection of tape Y with 100% methanol and 1% Triton X-100 pretreatment as the skin tape and permeabilization methods of choice respectively for advancement to the immunodiagnostic study of HD-adducted keratin on the tape.

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INTRODUCTION:

Keratin is an abundant structural resident protein of the stratum corneum of skin.[1, 2] When exposed to the alkylating effects of sulfur mustard (HD), keratin undergoes specific alkylational conformational changes of specific amino acids (i.e., glutamines, cysteines, asparagines) that result in the formation of characteristic and immuno-detectable HD keratin adducts.[3, 4] Recently monoclonal and polyclonal human antibodies to HD-induced keratin adducts have become available.[3] These antibodies combined with the investigative dermatological practice of skin tape-stripping present the possibility of noninvasively visualizing HD-adducted keratin. Routine diagnostic immunohistochemical procedures performed on the tape can then be conducted in advance of the presentation of characteristic HD-induced skin vesications.[5,6] Development of laboratory-based methods as summarized in this technical report is to satisfy proof-of-concept that stripped skin cells on tape can withstand a variety of staining paradigms. Further, it is to approach the promise of a noninvasive HD diagnostic strategy that is ultimately applicable for use in the field.

Based upon our earlier controlled laboratory-based immunohistochemical experiments of HD skin exposure[7, 8] and the expectations of available human antibody to HD-adducted keratins, it is projected that double-sided transparent tape can be used to strip superficial skin cells of the stratum corneum of animal skin, human skin explants and human skin exposed to vesicating doses of HD vapor *in vivo* and *in vitro.* Finally, removed skin cells that adhere to the stripping tape will be specifically stained by routine immunoperoxidase procedures performed on the tape to visualize HD-adducted keratin. This initial technical report summarizes the following: 1) the practice and use of tape-stripped normal human skin cells, 2) selection of an ideal tape that maintains its structural integrity, promotes adherence of cells, and resists background staining throughout selected protocol procedures, 3) modifications of a nonspecific counterstain to test cellular adhesion and cellular response, and 4) testing of pretreatments for fixation and permeabilization of cells on the tape.

MATERIALS AND METHODS:

Human Skin Tape Stripping: Double-sided optically clear tapes, Y and Z, and medical grade double-sided tape, A, were purchased from Light Fabrications, Inc.[9] Tapes were cut into one-centimeter squares and adhered to 1 x 3 microscope glass slides. The volar surface of forearms of willing investigators were cleaned with sterile alcohol pads and air dried. Backing from slide tapes was removed and the whole slide pressed firmly on the forearm for about 10 seconds. Slides were carefully removed at a 45-degree angle to ensure even cell adherence to the tape's adhesive surface. The slides with attached cells were subjected to different experimental staining and handling paradigms identified below. After each experiment, slides were cover-slipped with permount mounting media and a 24 mm x 30 mm cover glass. Slides were dried overnight and photomicrographed using an Olympus Vanox light microscope fitted with a Nikon Digital Sight® camera.[10] Finalized pictures were adjusted by Adobe Photoshop Elements®, using white point level adjusters.[11]

Nonspecific Staining: To determine a broad spectrum of effects, all tapes were subjected to six different staining protocols for differing time periods with and without hotplate heating. Staining

was performed with either sequential applications of staining ingredients or with premixed solutions of staining ingredients: 1) 100% methanol pretreatment + sequential stain application, 2) 100% methanol pretreatment + premixed stain application, 3) 100% acetone pretreatment + sequential stain application, 4) 100% acetone pretreatment + premixed stain application, 5) 1% Triton-X-100 + sequential stain application, 6) 1% Triton-X-100 + premixed stain application. Pretreatments with methanol, acetone and Triton-X-100 were conducted for 10 minutes at room temperature followed by a rinse in Millipore deionized water. Control slides received phosphate buffered saline (PBS) pretreatment + sequential stain or premixed stain application. For all sequential stain applications a 1:1 mixture of methylene blue/azure II was applied to the slide for 30 seconds, rinsed with deionized water and air dried. After drying, a 2:1 ratio of sodium borate and basic fuchsin was applied for 30 seconds followed by a rinse with Millipore deionized water and air drying. All solutions were dispensed through a 0.22 µm Millipore filter affixed to a 10 cc syringe. For all premixed applications, one part methylene blue, one part azure II, one part basic fuchsin and two parts sodium borate were premixed and dispensed through a 10 cc syringe. This stain mixture was applied to tapes for one minute then rinsed with Millipore deionized water, air dried and cover-slipped. Selected slides were heated during staining procedures by placing the slide on a hotplate for 30 seconds.

RESULTS:

Medical grade double-backed tape, A, stood up poorly to all procedures (Fig. 1). Backgrounds were heavily stained, and adhesion between tape and cell seemed to be lost after pretreatment. Many of the remaining cells on tape A were folded, creating deep dye pockets. On the other hand, optically clear tapes Y and Z presented clean, clear backgrounds with vividly dyed cells (Figs. 2, 3, 4, 5). The integrity of adhesion between the tape and the cells seemed undiminished after pretreatments. Cells appeared flat with well demarcated edges. Most staining procedures produced vivid blue stains with consistently clear backgrounds. Methanol- and Triton-X-100-pretreated tapes appeared more uniformly dyed than those pretreated with acetone and PBS, while cells pretreated with Triton-X-100 were consistently superior to those with methanol pretreatment. In all cases, the use of a hotplate presented more uniform vivid staining and consistent morphological presentations. Results with basic fuchsin in staining sequences were inconsistent.

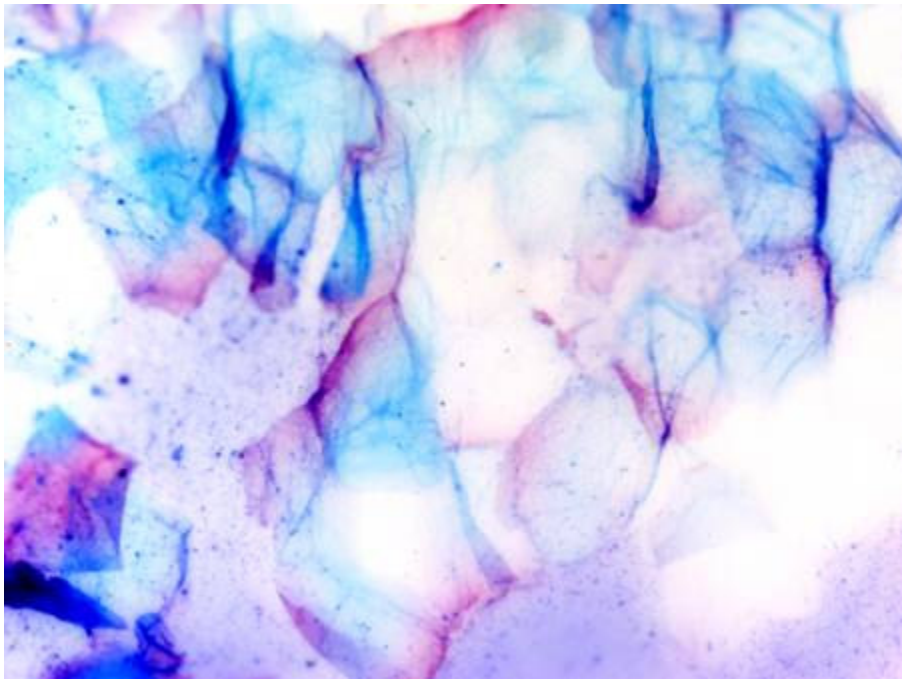

Figure 1: Typical appearance of stripped cells on tape A. Microscopic magnification 60x.

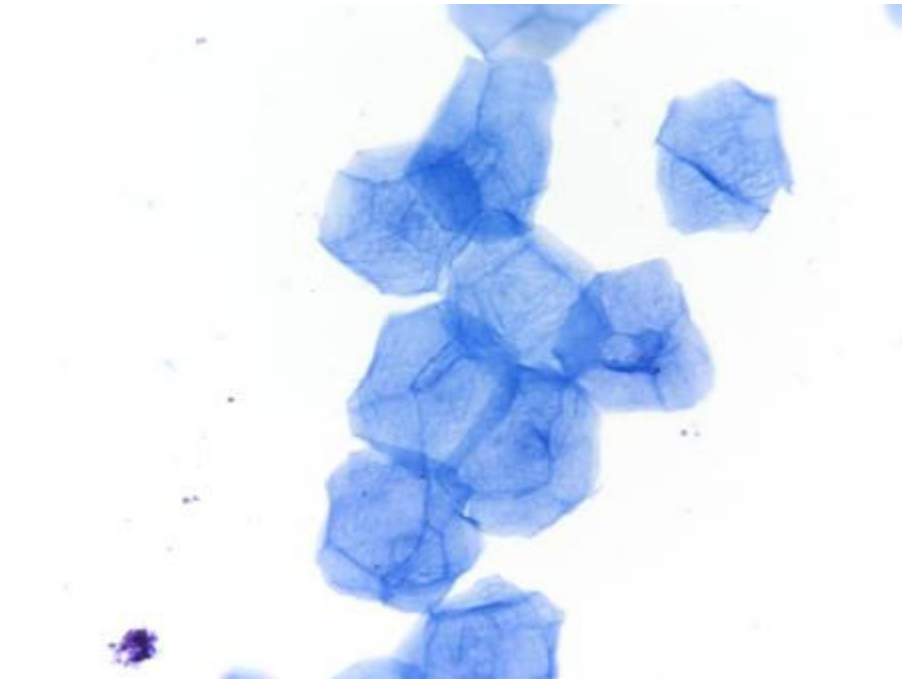

Figure 2: Stripped cells on tape Y without pretreatment. Methylene blue and azure II stain performed on hotplate. Microscopic magnification 60x.

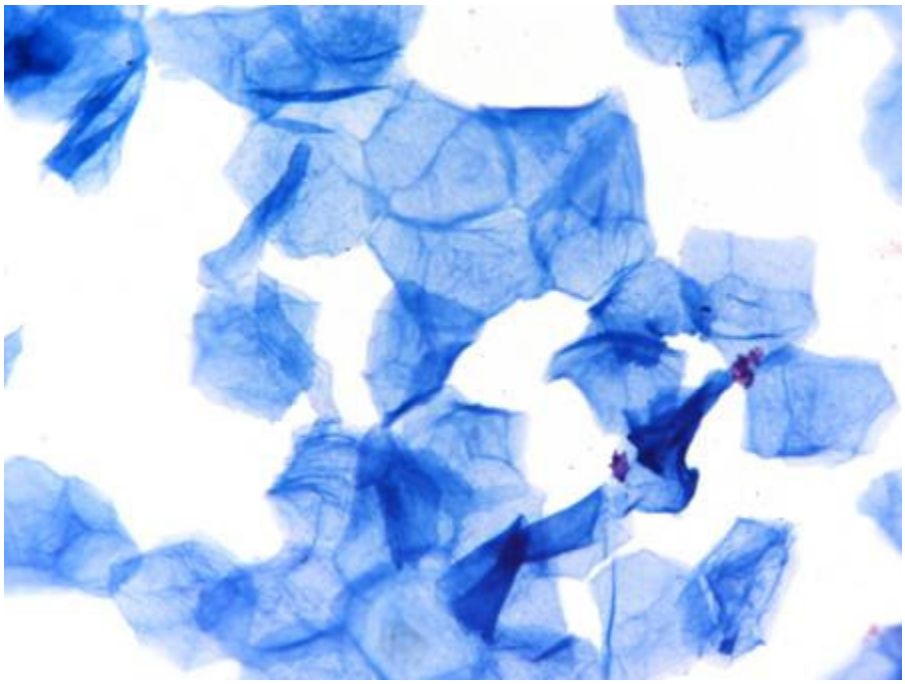

Figure 3: Stripped cells on tape Y with 1% Triton-X-100 pretreatment. Methylene blue and azure II stain performed on hotplate. Microscopic magnification 60x.

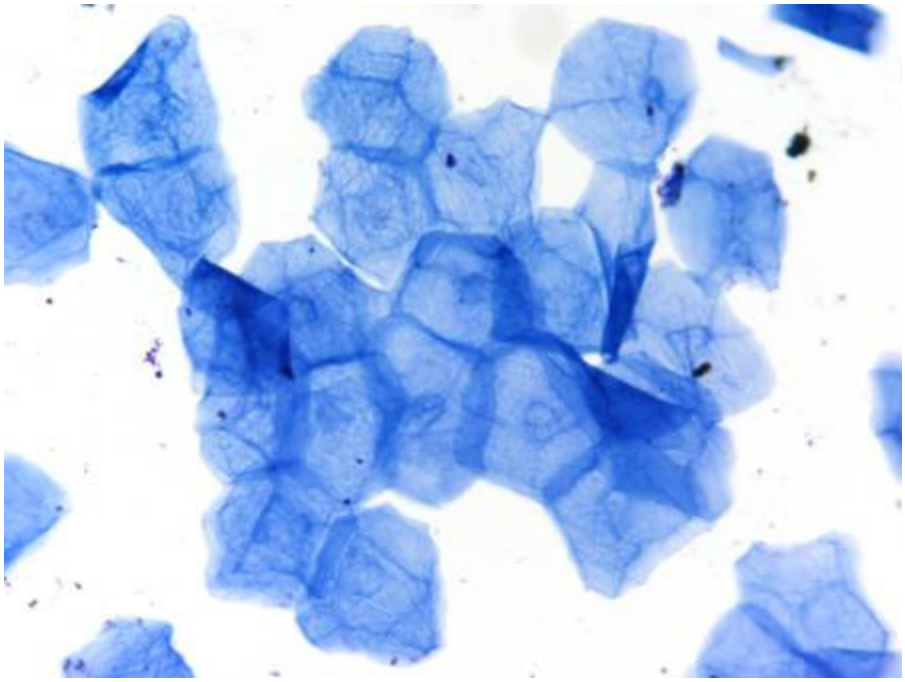

Figure 4: Stripped cells on tape Z without pretreatment. Methylene blue and azure II stain performed on hotplate. Microscopic magnification 60x.

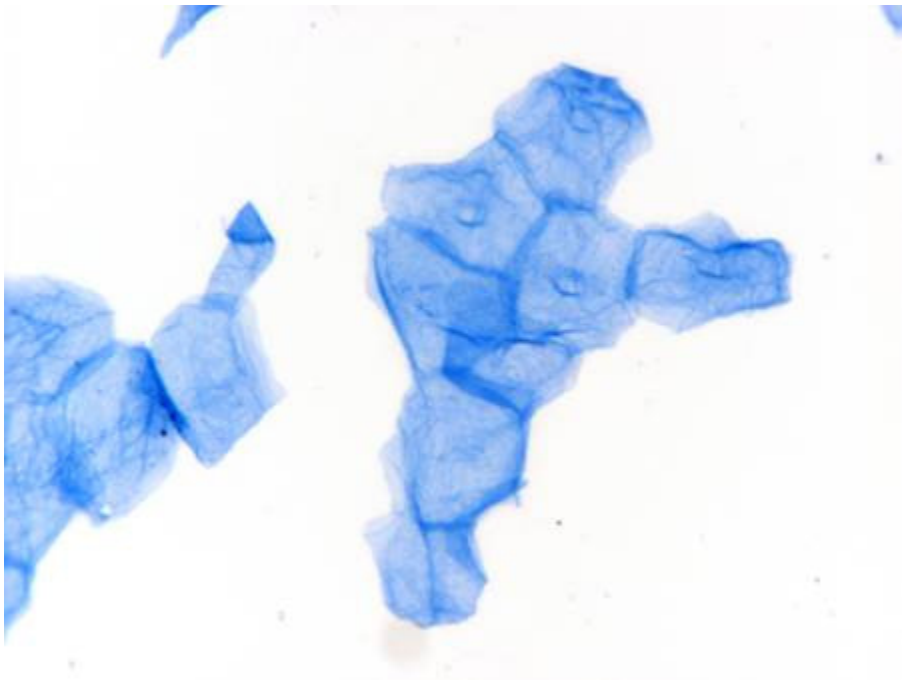

Figure 5: Stripped cells on tape Z with 100% methanol pretreatment. Methylene blue and azure II stain performed on hotplate. Microscopic magnification 60x.

DISCUSSION AND CONCLUSION:

The results of this morphological technical study demonstrate that tapes Y and Z are superior to tape A for nonspecific staining, microscopic clarity and morphologic integrity. Tape Y is less expensive and more readily available from the manufacturer than tape Z, making tape Y the more practical tape of choice for these studies. Permeabilization and fixation pretreatment experiments with tape Y show that 100% methanol and 1% Triton-X-100 are superior to 100% acetone and PBS for cellular morphological and staining presentations with little effect on cell adhesion, tape integrity, or image capturing. At this time, based upon this series of experiments the tape of choice for planned subsequent noninvasive immunodiagnostic/confirmatory study of HD skin exposure is optically clear tape Y with either 100% methanol or 1% Triton-X-100 as pretreatment. As expected most cells adhering to the tapes were cells of the stratum corneum with occasional cells of the stratum granulosum as recognized by cytoplasmic keratohyaline granules. Since these were human skin peels of human subjects, there was no mechanism to determine actual epidermal depth of repeated peels as might be approachable with animal skin study. Although skin tape peels have been used in dermatological practice and specific investigative study for sometime,[12] the particular results of the present laboratory-based technical study now add assurances that on the tape noninvasive immunodiagnosis of sulfur mustard skin exposure is feasible.

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